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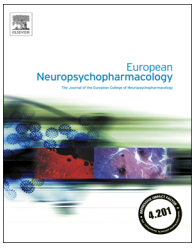

ASP5736, a novel 5-HT$_{5A}$ receptor antagonist, ameliorates positive symptoms and cognitive impairment in animal models of schizophrenia

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Abstract
We recently identified ASP5736, (*N*-(diaminomethylene)-1-(3,5-difluoropyridin-4-yl)-4-fluoroisoquinoline-7-carboxamide (2*E*)-but-2-enedioate), a novel antagonist of 5-HT$_{5A}$ receptor, and here describe the in vitro and in vivo characterization of this compound. ASP5736 exhibited a high affinity for the human 5-HT$_{5A}$ receptor (K_i=3.6±0.66 nM) and antagonized 5-carboxamidotryptamine (5-CT)-induced Ca^{2+} influx in human cells stably expressing the 5-HT$_{5A}$ receptor with approximately 200-fold selectivity over other receptors, including other 5-HT receptor subtypes, enzymes, and channels except human 5-HT$_{2c}$ receptor (Ki=286.8 nM) and 5-HT$_7$ receptor (Ki=122.9 nM). Further, ASP5736 dose-dependently antagonized the 5-CT-induced decrease in cAMP levels in HEK293 cells stably expressing the 5-HT$_{5A}$ receptor. We then evaluated the effects of ASP5736 on cognitive impairments in several animal models of schizophrenia. Working memory deficit in MK-801-treated mice and visual learning deficit in neonatally phencyclidine (PCP)-treated mice were both ameliorated by ASP5736. In addition, ASP5736 also attenuated MK-801- and methamphetamine (MAP)-induced hyperactivity in mice without causing sedation, catalepsy, or plasma prolactin increase. The addition of olanzapine did not affect ASP5736-induced cognitive enhancement, and neither the sedative nor cataleptogenic effects of olanzapine were worsened by ASP5736. These results collectively suggest that ASP5736 is a novel and potent 5-HT$_{5A}$ receptor antagonist that not only ameliorates positive-like symptoms but also cognitive impairments in animal models of schizophrenia,

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without adverse effects. Present studies also indicate that ASP5736 holds potential to satisfy currently unmet medical needs for the treatment of schizophrenia by either mono-therapy or co-administered with commercially available antipsychotics.

1. Introduction

Schizophrenia is a chronic, severe, and disabling psychiatric disease involving three major symptom classes (positive, negative, and cognitive). Cognitive impairments of schizophrenia have been increasingly focused because the cognitive impairments are known to be better predictors of functional outcome than indices from any other symptom domain such as positive symptom or negative symptom. (Green MF 1996, Hofer A et.al. 2005) However, no available antipsychotic has shown the clinically beneficial efficacy for the impairments. (Hill SK 2010)

Add-on pharmacological agents have been posited as potentially useful in relieving these as-yet untreated symptoms, as they can be used to modulate specific neurotransmitter systems hypothesized to be involved in cognitive functions. In the last decade, evidence has emerged that many cognitive domains are affected in schizophrenia, including attention, executive function, working memory, and visual and verbal learning and memory. Improvements in neurocognitive deficits have also been reported to be better predictors of social function than improvements in psychotic symptoms (Green, 2006).

The 5-HT$_{5A}$ receptor is a G-protein-coupled seven-transmembrane receptor and the human gene of this receptor was cloned in 1994 (Rees et al., 1994). However, few reports have touched on the function of the receptor due to a lack of specific ligands. Findings from 5-HT$_{5A}$ receptor mRNA localization (Rees et al., 1994, Pasqualetti et al., 1998) and immunolabelling studies (Oliver and Kinsey, 2000) have revealed widespread expression of this receptor in the central nervous system, including the cerebral cortex, hippocampus, nucleus accumbens, amygdala, and hypothalamus. In a number of these brain areas, the 5-HT$_{5A}$ receptor is expressed on neurons (e.g. on rat cortical and hippocampal pyramidal neurons) with little expression in peripheral tissues, which has provided insight into the potential roles of the receptor. In addition, increased exploratory behavior in novel environments displayed by 5-HT$_{5A}$ receptor KO mice compared to wild-type mice (Grailhe et al., 2001), together with the widespread localization pattern, has suggested that the 5-HT$_{5A}$ receptor is involved in mood, affective disorder, and cognitive function, while gene association studies imply that the 5-HT$_{5A}$ receptor may also play a role in schizophrenia and mood disturbance (Thomas, 2006, Jongen-Relo et al., 2006, Rueter et al., 2006).

ASP5736 (*N*-(diaminomethylene)-1-(3,5-difluoropyridin-4-yl)-4-fluoroisoquinoline-7-carboxamide (2*E*)-but-2-enedioate) (Figure 1) is a novel, potent and selective 5-HT$_{5A}$ receptor antagonist originally synthesized by Astellas Pharma Inc. Here, we evaluated the in vitro inhibitory effects of this compound on the 5-HT$_{5A}$ receptor, its affinity for various receptors, and its pharmacokinetics. We also evaluated the compound in animal models of positive-like symptoms, and cognitive impairment of schizophrenia. The efficacy and safety of ASP5736 was also tested in animal models in combination with an existing anti-psychotic, olanzapine, to determine if the drug can be used in combination with existing medicines.

2. Experimental procedures

2.1. Drugs and treatment

ASP5736, and phencyclidine hydrochloride (used as PCP) were synthesized at Astellas Pharma Inc. (Tsukuba, Japan). Olanzapine (brand name: Zyprexa) was purchased from Eli Lilly (Indianapolis, USA) and extracted at Astellas Pharma Inc. Methamphetamine hydrochloride (MAP) and (+)-MK-801 hydrogen maleate (MK-801) were purchased from Dainippon Sumitomo Pharma Co., Ltd. (Tokyo, Japan) and Sigma Aldrich (St. Louis, MO, USA), respectively. ASP5736 and olanzapine were suspended in 0.5% (w/v) methyl cellulose (MC) and PCP, MAP, and MK-801 in saline. All compounds were administered at 10 mL/kg in mice and 1 mL/kg in rats. ASP5736 was corrected for salt content.

2.2. Inhibition of 5-HT$_{5A}$ receptor

2.2.1. Affinity of ASP5736 for human and mouse 5-HT$_{5A}$ receptor

2.2.1.1. Acquisition of HEK293 cells stably expressing human 5-HT$_{5A}$ receptor. An open reading frame (ORF) of the human 5-HT$_{5A}$ receptor (GenBank AF498985) was first cloned from a human whole brain cDNA library and then inserted into a pCR2.1 vector (Life Technologies, Carlsbad, CA, USA), and *Escherichia coli* containing the plasmid was mass cultured. The human 5-HT$_{5A}$ receptor full-length cDNA sequence was then analyzed, inserted into a pCDNA3.1 recombinant expression vector (Life Technologies), and its plasmid was mass cultured. Human embryonic kidney-induced (HEK) 293 cells (American Type Culture Collection, Manassas, VA, USA) were seeded, and the aforementioned expression plasmid (1 μg) was added in combination with lipofectamine 2000 (Life Technologies; 2 μL), to introduce the gene. Geneticin (G418 sulfate 500 μg/mL;

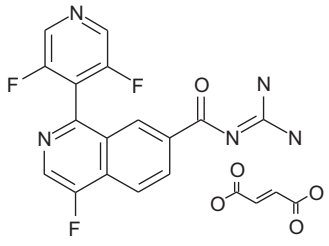

Figure 1 Chemical structure of ASP5736. *N*-(diaminomethylene)-1-(3,5-difluoropyridin-4-yl)-4-fluoroisoquinoline-7-carboxamide (2*E*)-but-2-enedioate.

Kanto Chemical Co., Inc., Tokyo, Japan) was used as a drug-resistant marker to screen cells for cells expressing the human 5-HT_{5A} receptor.

2.2.1.2. Inhibition of human 5-HT_{5A} receptor binding to test compounds. Cell membranes (25 μg of protein), [^{3}H]5-CT (5-carboxamidotryptamine) (1.5 nM at final concentration), and test compounds (0.03–30 μM at final concentration) were placed in 96-well plates and incubated for 60 min at 37 °C in incubation buffer (Tris(HCl) pH7.4 50 mM, $MgSO_4$ 10 mM, EDTA 0.5 mM). The reaction was terminated via rapid filtration through 96-well GF/C filter plates (PerkinElmer Inc.) pre-soaked in 0.3% polyethyleneimine for 30 min. Filter plates were washed 5 times with **50 mM Tris HCl at pH 7.4** and dried. Liquid scintillant (30 μL; MicroScint™-20, PerkinElmer Inc., Waltham, MA, USA) was placed on the filters, and the radioactivity was measured using a TopCount (PerkinElmer Inc.). Nonspecific binding was determined with 1.5 μM 5-CT.

Values of the apparent equilibrium dissociation constant of inhibitors (K_i) were calculated using SAS version 8.2 (SAS Institute Inc., Cary, NC, USA). Mean and standard error of the mean (SEM) were obtained from three independent experiments. To test the inhibitory effect following the binding of [^{3}H]5-CT to the compound in each experiment, the IC_{50} value was calculated by measuring radioactivity when only DMSO was added as (0% inhibition), and when 1.5 μM 5-CT was added (100% inhibition). All other K_i values were calculated using the following equation:

$$K_i = IC_{50}/(1 + \text{Concentration of the ligands added}/Kd)$$

The Kd value of [^{3}H]5-CT was determined with non-linear regression analysis.

2.2.2. Antagonistic effect of ASP5736 on human 5-HT_{5A} receptor

We established HEK293 cells that stably expressed the human 5-HT_{5A} receptor and G15α, and examined the effect of ASP5736 on 5-CT-induced Ca influx using a fluorescence imaging plate reader (FLIPR) (Molecular Devices, Sunnyvale, CA, USA) (Noda et al. 2003). HEK293 cells harvested on a 384-well plate were incubated with assay buffer (1 × Hank's balanced salt solution (Life Technologies), 20 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) (Sigma-Aldrich)) containing 1 μM 1-[2-amino-5-(2,7-difluoro-6-acetoxymethoxy-3-oxo-9-xanthenyl)phenoxy]-2-(2-amino-5-methylphenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, tetra(acetoxymethyl) ester (Fluo 4-AM) (Dojindo, Kumamoto, Japan), 0.01% F-127 pluronic acid (Life Technologies), and 625 μM probenecid (Sigma-Aldrich) for 1.5 h. ASP5736 (0.0125–1250 nM) dissolved in assay buffer containing 0.02% CHAPS (Nacalai Tesque, Inc., Kyoto, Japan) was applied for 15 min, and then 62.5 nM 5-CT was applied. Ca influx was monitored with FLIPR^{tetra}, and differences were analyzed using ScreenWorks™ software (Molecular Devices).

2.3. Antagonistic effect of ASP5736 on 5-CT-induced decrease in cAMP levels in cells stably expressing human 5-HT_{5A} receptor

Intracellular cAMP content was measured with GloSensor™ cAMP Assay (Promega, Fitchburg, WI, USA). Native or 5-HT_{5A}-expressing HEK293 cells were transfected with GloSensor™ plasmid using Lipofectamine 2000 (Life Technologies) in Opti-MEM (Life Technologies). After 24 h, cells were incubated in assay buffer (1 × HBSS and 20 mM HEPES) containing 2% luciferin (Promega) at 37 °C for 1 h and room temperature for 1 h. ASP5736 and 1 μM forskolin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) were treated in the presence of 50 nM SB269970, a selective 5-HT_7 antagonist (Hagan et.al, 2000, Peter et al., 2000), for 15 min. 5-CT (30 nM) was applied for 30 min, and luminescence was monitored using FLIPR^{tetra} in luminescence mode. Area under the curve (AUC) was calculated using ScreenWorks™ software (Molecular Devices).

2.4. Affinity for multiple receptors, ion channels and transporters

To determine the affinity of ASP5736 for a wide range of receptors, transporters and ion channels, initial receptor binding screens (62 assays) were conducted with duplicate samples of 1 μM ASP5736 by Sekisui Medical Inc. (Tokyo, Japan) using proprietary assay formats.

2.5. Animals

Mice were housed in groups of 10 in temperature- and humidity-controlled rooms (23 ± 1 °C and 55 ± 5%) under a 12-h light-dark cycle with water and laboratory chow available ad libitum. All animals were experimentally naive and used only once. All in vivo experimental procedures were performed under lit conditions. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Astellas Pharma Inc. Further, the Astellas Pharma Inc. Tsukuba Research Center was awarded Accreditation Status by the AAALAC International. All efforts were made to minimize the number of animals used and to avoid suffering and distress.

2.6. Pharmacokinetic study in mice

Experiments were performed using five-week-old male ddY mice (Japan SLC, Inc., Hamamatsu, Japan), which had been freely feeding. In the first examination, animals received an oral (p.o.) administration of ASP5736 (0.03 mg/kg) (suspended in 0.5% MC) and were kept in rearing cages. Under isoflurane anesthesia, the animals were sacrificed, and whole blood samples were taken using a syringe containing heparin sodium at 0.5, 1, and 2 h post-dose. Given that a above experiment revealed the T_{max} of this compound to be 1 h, animals received an oral administration of ASP5736 (0.01 mg/kg) and were sacrificed under isoflurane anesthesia at 1 h post-dose, and whole blood samples were taken using a syringe containing heparin sodium. After collection of whole blood, the cerebrum was also removed from each animal. Whole blood samples were centrifuged to separate plasma. Plasma and cerebrum were stored at −20 °C until assay.

For the analysis, 25 μL plasma was mixed with 25 μL 50% acetonitrile and 200 μL acetonitrile containing internal standard (IS; final: 20 ng/mL diazepam). The mixture was subjected to centrifugation at 2150*g* for 10 min, and the resulting supernatant was injected into a LC-MS/MS system. This consisted of the Prominence HPLC system (Shimadzu, Kyoto, Japan) with an XTerra MS C18 column (4.6 × 50 mm^2, 5 μm particle size, Waters, Milford, MA, USA) and an API4000 LC/MS/MS instrument (AB SCIEX, Framingham, MA, USA). Data were processed using Analyst software (AB SCIEX, Ontario, Canada), and the concentration of ASP5736 was calculated via an internal standard.

2.7. Animal models of schizophrenia

2.7.1. MK-801-induced working memory deficit in mice during Y-maze test

Spatial working memory performance was assessed by recording spontaneous alternation behavior of male ddY mice (aged 5-6 weeks, Japan SLC, Inc.) in a Y-maze as previously described (Maurice and Privat, 1997). The maze was constructed from gray polyvinyl chloride, with arms (length, 40 cm; height, 13 cm; width at bottom, 3 cm; and width at top, 10 cm) converging at equal angles. Thirty minutes after p.o. administration of ASP5736, olanzapine, or vehicle, 0.15 mg/kg of MK-801 was administered

intraperitoneally (i.p.) to each animal. Control animals were administered vehicle instead of ASP5736 and saline instead of MK-801 (i.p.). Twenty minutes after administration of MK-801 or saline, each mouse was placed at the end of one arm and allowed to freely explore the apparatus for 8 min. The total number of arm entries was recorded for each animal throughout the period. Alternation was defined as entries into all three arms on consecutive occasions. The alternation rate was calculated using the following formula:

$$\text{Alternation rate}\,(\%) = 100 \times \text{Number of alternations}/(\text{Number of total arm entries} - 2)$$

2.7.2. Novel object recognition test (NORT) in neonatally PCP-treated mice

Three-day-old male ddY mice (Japan SLC, Inc.) were housed 10–12 per cage with a stepmother. Saline or PCP (15 mg/kg) was administered subcutaneously once daily on days 7, 9, and 11 after birth. The mice were separated from their mother at 3 weeks of age, and used for NORT at 8–9 weeks old. Neonatal mice were treated with PCP, and the NORT was conducted as previously described (Harada et al., 2012).

The NORT consists of three different sessions: habituation, training, and testing. Each mouse was individually habituated to the box for 5 min in the absence of objects (habituation session). After 3–4 h, two different objects (objects A and B) were placed symmetrically 10 cm away from the two opposite corners of the back wall. The objects A and B were always in the same location. The animal was placed into the box facing a side-wall and allowed to explore the box for 5 min (training session). ASP5736 and olanzapine were orally administered about 60 and 50 min before the training session, respectively. Control animals were administered with vehicle instead of ASP5736. After the training session, the mouse was placed back in its home cage. After a 24 h retention interval, the object B was replaced with a novel object (object C.) The animal was placed back into the same box now with one familiar object (object A) and one novel object (object C), and allowed to explore the box for 5 min (testing session). The time spent exploring each object during the training and testing sessions was recorded. Exploration was defined as inquisitive activity directed toward the object, such as touching it with the nose or limbs, or sniffing the object. Exploratory preference was determined to assess cognitive function using the following formula:

$$\text{Exploratory preference}\,(\%) = 100 \times (\text{Times pent exploring novel object})/(\text{Total time exploring both objects})$$

The researcher was blind to the treatment condition of mice.

2.7.3. Spontaneous activity in mice

Five-week-old male ICR mice administered ASP5736, olanzapine, or vehicle (p.o.) were placed individually in experimental cages ($L \times W \times H$: $30 \times 35 \times 17.5$ cm^3), and their locomotor activity was recorded for 60 min via SUPERMEX (Muromachi Kikai Co. Ltd., Tokyo, Japan).

2.7.4. MAP-induced hyperactivity in mice

Five-week-old male ICR mice administered ASP5736, olanzapine, or vehicle (p.o.) were individually placed into experimental cages ($L \times W \times H$: $30 \times 35 \times 17.5$ cm^3). After 30 min, each mouse received a subcutaneous (s.c.) administration of MAP (1.5 mg/kg), were individually replaced into experimental cages, and their locomotor activity was recorded for 60 min via SUPERMEX (Muromachi Kikai Co. Ltd.).

2.7.5. Catalepsy in mice

Five-week-old male ICR mice were administered ASP5736, olanzapine, or vehicle (p.o.). After 60 min, each mouse was assessed for catalepsy for 120 s. Catalepsy was measured via the bar method, which consists of placing an animal following drug administration, with its front legs resting on a bar suspended above the floor of the test apparatus. Intensity of catalepsy was measured as the length of time the test subject maintains this abnormal posture. Catalepsy time (duration of catalepsy) was calculated.

2.7.6. Plasma prolactin concentration in mice

Experiments were performed using nine-week-old male ICR mice (Japan SLC, Inc., Hamamatsu, Japan) which had been freely feeding. ASP5736 and risperidone (0.1 mg/kg) were orally administered (by 0.5% MC), and mice were kept in rearing cages. Under isoflurane anesthesia, animals were sacrificed, and whole blood samples were taken using a syringe containing heparin sodium at 1 or 0.5 h post-dose, respectively. Whole blood samples were centrifuged to separate plasma. Plasma was stored at −20 °C until assay. Plasma prolactin levels were measured using an enzyme-linked immunosorbent assay (ELISA; Calbiotech, Spring Valley, CA, USA) with a sensitivity of 0.2 ng/mL.

2.8. Co-administration with olanzapine in animal models of schizophrenia

2.8.1. Effect of ASP5736 and olanzapine on MK-801-induced working memory deficit in mice during Y-maze test

Methods were the same as in Section 2.7.1. MK-801 (0.15 mg/kg, i.p.) was administered to each animal 30 min after administration of ASP5736 or vehicle with olanzapine (1 mg/kg, p.o.).

2.8.2. Effect of ASP5736 and olanzapine on spontaneous activity in mice

Methods were the same as in Section 2.7.3. Olanzapine or vehicle was administered in combination with ASP5736 (0.003 mg/kg, p.o.) to 5-week-old male ICR mice that were then individually placed into experimental cages with locomotor activity recorded for 60 min via SUPERMEX.

Table 1 In vitro 5-HT$_{5A}$ receptor binding affinity and 5-CT-induced Ca influx in 5-HT$_{5A}$ receptor

	IC$_{50}$ (nM)	Ki (nM)	Reference	
			Compound	IC$_{50}$ (nM)
Human 5-HT$_{5A}$ receptor	4.6±0.82	3.6±0.66	Methiothepin	3.3±0.52
5-CT-induced Ca influx	1.0±0.09	-	Methiothepin	2900±420

Data represent mean±SEM of three independent experiments.

2.8.3. Effect of ASP5736 and olanzapine on MAP-induced hyperactivity in mice

Methods were the same as in Section 2.7.4. Olanzapine or vehicle was administered in combination with ASP5736 (0.003 mg/kg, p.o.) to 5-week-old male ICR mice that were individually placed into experimental cages with locomotor activity recorded for 60 min via SUPERMEX.

2.8.4. Effect of ASP5736 on catalepsy in mice with olanzapine

Methods were the same as in Section 2.7.5. Olanzapine or vehicle was administered to 5-week-old male ICR mice with ASP5736 (0.003 mg/kg, p.o.). After 60 min, each mouse was assessed for catalepsy for 120 sec.

2.9. Statistical analysis

All values are expressed as mean±standard error of the mean (SEM). Values for IC_{50} were determined by fitting the data to a logistic equation using SAS software (SAS Institute Inc., Cary, NC, USA). Student's *t*-test was used for statistical analysis of control (vehicle- and saline-treated) group vs. vehicle- and MAP-treated group, vehicle- and MK-801-treated group and vehicle- and PCP-treated group. Dunnett's multiple comparison test was used to compare multiple groups. For all tests, $P<0.05$ was considered significant.

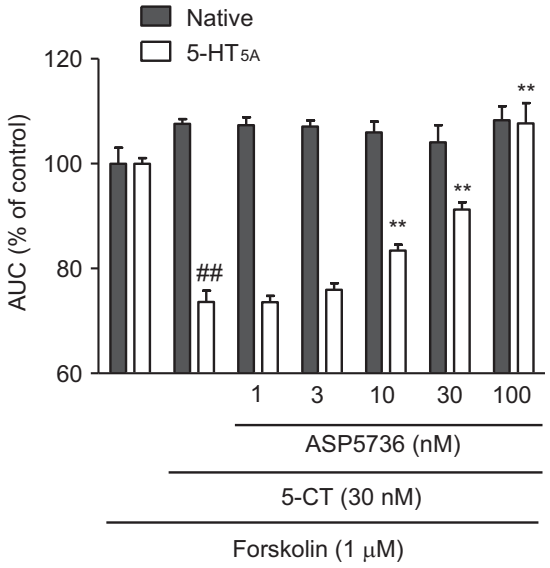

Figure 2 Intracellular cAMP concentration in cells stably expressing 5-HT_{5A} receptor. cAMP levels were decreased by 5-CT (30 nM), a non-selective 5-HT_{5A} agonist, in cells stably expressing 5-HT_{5A} receptors. ASP5736 dose-dependently antagonized this decrease. In contrast, in native cell, cAMP didn't change by 5-CT or ASP5736. All data are mean±SEM ($n=4$ for each time point). $^{\#\#}P<0.01$, statistically significant compared with vehicle-treated forskolin (1 μM) group (Student's *t*-test). $^{**}P<0.01$, statistically significant compared with vehicle-treated 5-CT group (Dunnett's multiple comparison test).

3. Results

3.1. Binding to 5-HT_{5A} receptor and blocking of function

ASP5736 bound to the human 5-HT_{5A} receptor with a Ki value of 3.7±0.66 nM. ASP5736 inhibited 5-CT-induced Ca^{2+} influx using cells stably expressing both 5-HT_{5A} receptor and G15α (as determined using 5-HT_{5A} receptor functional screening of compounds) with an IC_{50} value of 1.0±0.09 nM. K_i and IC_{50} values for ASP5736 are summarized in Table 1. Further, ASP5736 antagonized the 5-CT-induced decrease in cAMP in cells stably expressing the human 5-HT_{5A} receptor in a dose-dependent manner (Figure 2).

3.2. Plasma and brain concentration studies in mice

ASP5736 concentration was observed in plasma and whole brain in mice at 0.5, 1, and 2 h following oral administration of the compound (0.03 mg/kg) as shown in Table 2, suggesting that ASP5736 is orally absorbable and CNS penetrable. As the T_{max} of ASP5736 (0.03 mg/kg, p.o.) in the brain ranged from 1 to 2 h, we adopted a 60-min post-administration period for subsequent pharmacology studies. Further, brain concentrations with 0.01 and 0.03 mg/kg were nearly dose-proportional.

3.3. Affinity for multiple receptors, ion channels and transporters

The affinity of ASP5736 for 54 receptors, 7 ion channels, and 4 transporters was evaluated using a radioligand binding panel screen. At a concentration of 1 μM, ASP5736 did not inhibit ligand binding to any target by more than 50%, except for human 5-HT_{2c} receptor and 5-HT_7 receptors (Table S-1). Subsequent concentration-response experiments showed that ASP5736 inhibited [^{3}H]-5-HT binding to human 5-HT_{2c} receptor and 5-HT_7 receptors with Ki value of 286.8 nM and 122.9 nM, respectively.

Table 2 Plasma and brain concentration of ASP5736 in mice

ASP5736	0.01 mg/kg, p.o.			0.03 mg/kg, p.o.		
	Plasma (ng/mL)	Brain (ng/g)	Kp brain	Plasma (ng/mL)	brain (ng/g)	Kp brain
0.5 h	-	-	-	10.8±1.7	1.2±0.6	0.11
1 h	5.6±1.0	0.5±0.1	0.08	9.5±1.0	1.5±0.8	0.15
2 h	-	-	-	10.4±0.5	1.5±0.8	0.14

Data expressed as mean±SEM of duplicate samples ($n=3$).

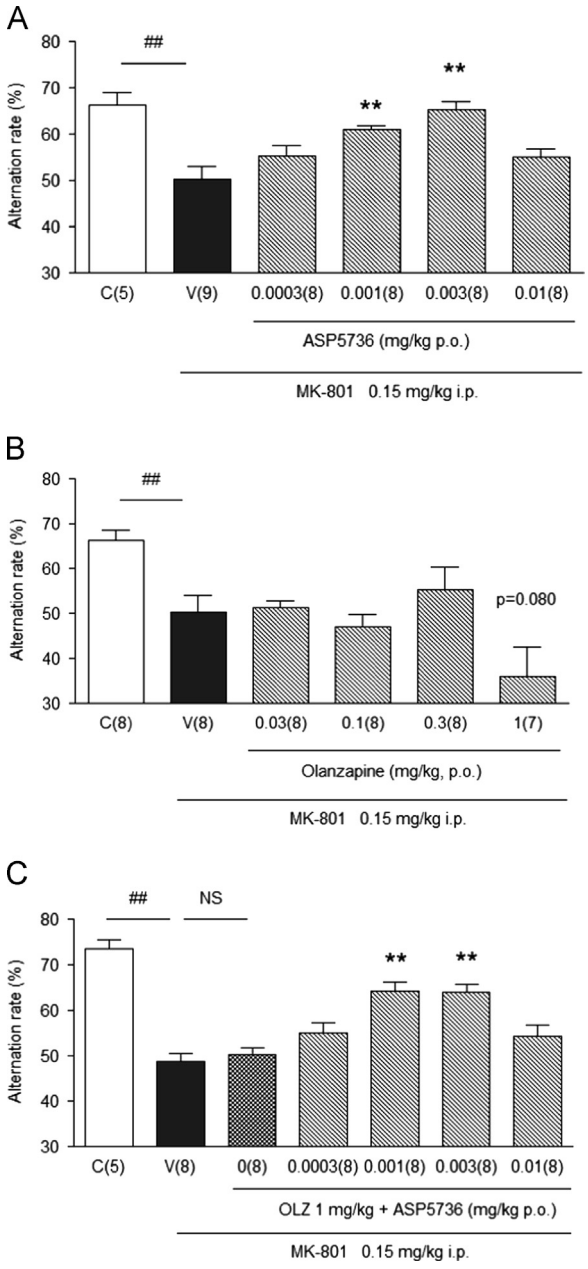

Figure 3 Effect of ASP5736 and olanzapine on MK-801-induced working memory deficit in mouse Y-maze test. (A) Effect of ASP5736 alone. $^{\#\#}P<0.01$, statistically significant compared with control (vehicle- and saline-treated) group (Student's *t*-test). $^{**}P<0.01$, statistically significant compared with vehicle-treated MK-801 group (Dunnett's multiple comparison test). (B) Effect of olanzapine alone. $^{\#\#}P<0.01$, statistically significant compared with control (vehicle- and saline-treated) group (Student's *t*-test). (C) Effect of ASP5736 in combination with olanzapine. The dose of olanzapine was determined from the inhibition dose (ID_{50} value) of MAP induced-hyperactivity. C, control (vehicle- and saline-treated); V, vehicle (vehicle- and MK-801-treated); and 0, 0 mg/kg ASP5736 (olanzapine 1 mg/kg-MK-801). $^{\#\#}P<0.01$, statistically significant compared with control (vehicle- and saline-treated) group (Student's *t*-test). $^{**}P<0.01$, statistically significant compared with olanzapine 1 mg/kg-treated MK-801 group (Dunnett's multiple comparison test). Each value shows the mean $\pm$ SEM ($n=8$ for each group).

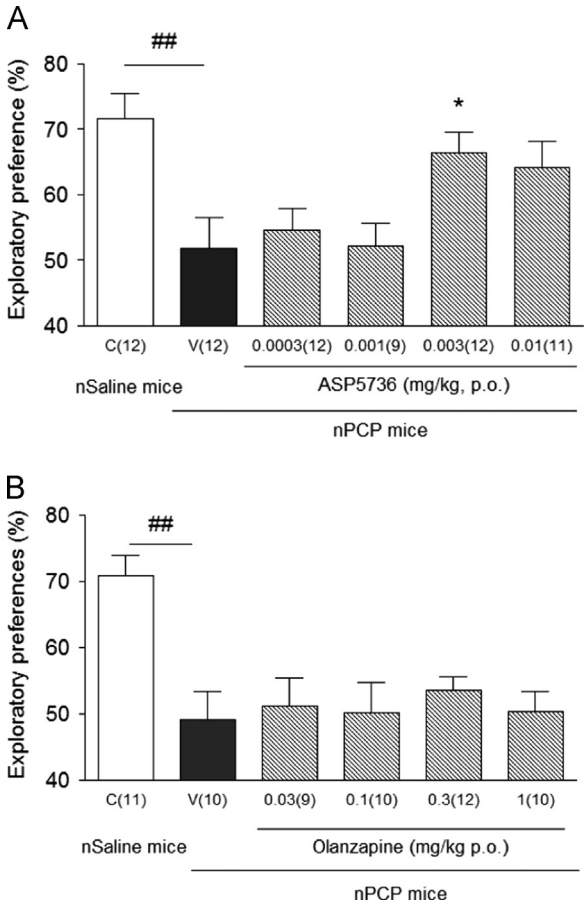

Figure 4 Effect of ASP5736 and olanzapine on neonatal PCP treatment-induced learning deficit in mice during novel object recognition test. Saline or PCP (15 mg/kg) was subcutaneously administered at postnatal days 7, 9, and 11. Exploratory preference for novel object in the testing session was measured. Effects of ASP5736 (A) and olanzapine (B) are shown. Values shown represent mean $\pm$ SEM. Number in parenthesis indicates number of mice in each group. $^{\#\#}P<0.01$, statistically significant compared with control neonatal saline group (Student's *t*-test). $^{*}P<0.05$, statistically significant compared with vehicle-treated neonatal PCP group (Dunnett's multiple comparison test).

3.4. Evaluation of ASP5736 in animal models of schizophrenia

3.4.1. MK-801-induced working memory deficit in mouse Y-maze test

We examined the effect of ASP5736 on working memory disruption in mice based on observation of spontaneous alternation behavior in a Y-maze with MK-801. We also evaluated the effect of this compound in combination with olanzapine on spontaneous alternation behavior. Administration of MK-801 consistently induced a marked decrease in the alternation rate in the Y-maze test in each experiment ($P<0.001$, by Student's *t*-test), as coinciding with previous results (Parada-Turska and Turski, 1990). ASP5736 significantly attenuated the MK-801-induced decrease in alternation rate, and statistical analysis revealed that the effect of

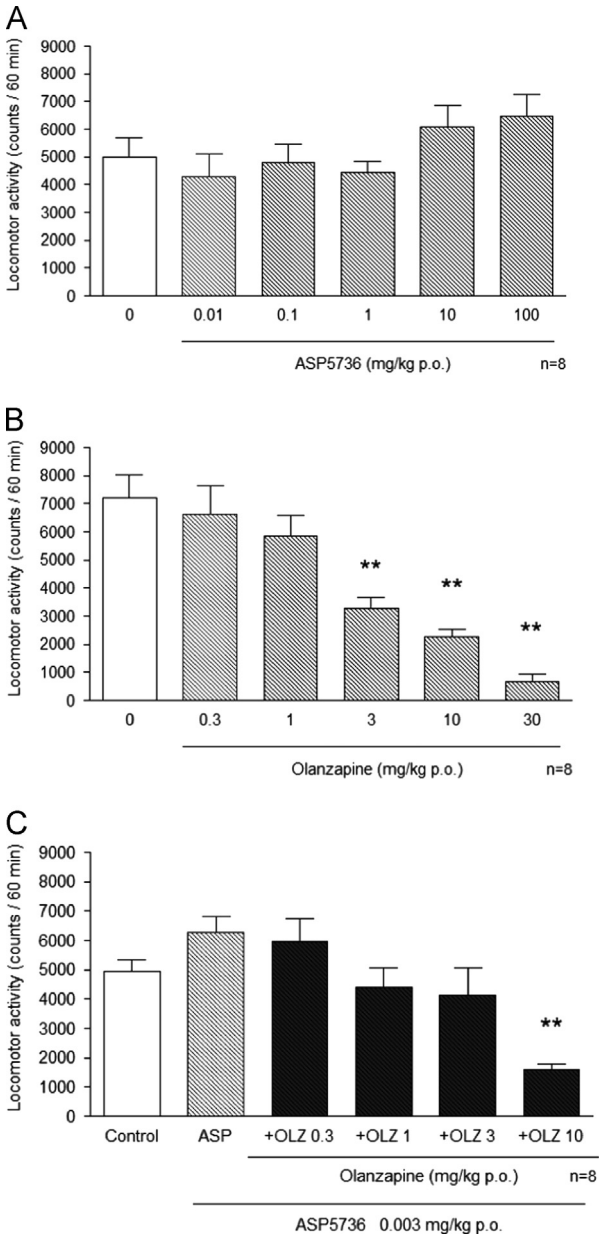

Figure 5 Effect of ASP5736 and olanzapine on spontaneous activity in mice. (A) Effect of ASP5736 alone. ASP5736 did not affect spontaneous activity at any doses. (B) Effect of olanzapine alone. $^{**}P<0.01$, statistically significant compared with vehicle-treated group (Dunnett's multiple comparison test). (C) Effect of olanzapine in combination with ASP5736. We determined the dose of ASP5736 from the effective dose of MK-801-Y-maze and nPCP-NORT study. Control, vehicle-treated, OLZ, olanzapine and ASP, ASP5736 0.003 mg/kg-treated. $^{**}P<0.01$, statistically significant compared with ASP (vehicle- and ASP5736 0.003 mg/kg-treated) group (Dunnett's multiple comparison test). Each value represents mean $\pm$ SEM ($n=8$ for each group).

ASP5736 was significant at doses of 0.001 and 0.003 mg/kg (p.o.) (Figure 3A).

We then evaluated the effect of ASP5736 on spontaneous alternation behavior in combination with olanzapine at 1 mg/kg (p.o.), which was the dose selected based on the potency of inhibitory effect on MAP-induced hyperlocomotion in our preliminary study. Olanzapine alone had no effect on MK-801-induced spontaneous alternation behavior deficit (Figure 3B). However, ASP5736 in combination with olanzapine still significantly attenuated the spontaneous alternation behavior deficit as the same degree as ASP5736 alone (Figure 3C).

Furthermore, we also evaluated the effect of ASP5736 on spontaneous alternation behavior in treatment (MK-801)-free mice in separate experiments. The compound did not affect the alternation rate (Table S-3).

3.4.2. Neonatal PCP treatment-induced learning deficit in mouse NORT

The NORT task is a model for assessing visual-recognition memory which takes thadvantage of the natural preference of mice for novelty (mice that recognize a familiar object will instinctively spend more time exploring novel objects) (Ennaceur and Delacour, 1988). Neonatal PCP treatment impairs the ability to perform this task in adults (Harada K. et al., 2012). Here, we evaluated the effect of ASP5736 on neonatal PCP-induced deficits in mice. No particular preference was noted for either of two objects presented in the training session (data not shown). However, while control mice (treated neonatally with saline) spent nearly 70% of total time exploring the novel object in the test session, mice treated neonatally with PCP spent approximately only 50% of total time exploring the novel object, a significant difference compared with control mice ($P<0.01$, by Student's t-test) (Figure 4A and B). This finding suggests that neonatal PCP treatment did indeed impair the cognition of mice in the NORT. ASP5736 increased the exploratory preference in neonatally PCP-treated mice and significantly improved memory impairment at 0.003 mg/kg (p.o.) (Figure 4A). In contrast, olanzapine did not change the exploratory preference in neonatally PCP-treated mice (Figure 4B).

3.4.3. Effect of ASP5736 on spontaneous locomotor activity in mice

Antipsychotic drugs are known to decrease spontaneous activity. In the present study, olanzapine decreased spontaneous activity in a dose-dependent manner in mice (Figure 5B). We examined the effect of ASP5736 on spontaneous activity in mice, and also evaluated the effects of this compound in combination with olanzapine on spontaneous activity. As shown in Figure 5A, ASP5736 exerted no decrease in spontaneous activity from 0.01 to 100 mg/kg (p.o.) in mice. Further, the inhibitory effect of olanzapine on spontaneous activity was not enhanced by ASP5736 at 0.003 mg/kg (Figure 5C), the dose at which MK-801-induced spontaneous alternation deficit was ameliorated.

3.4.4. Effect of ASP5736 on MAP-induced hyperactivity in mice

Figure 6A and B show the effects of ASP5736 and olanzapine on MAP-induced hyperactivity in mice, respectively. MAP-treated mice showed significant hyperactivity compared with control (vehicle-saline treated) mice, as shown in Figure 6 ($P<0.001$, by Student's t-test). Treatment with ASP5736 significantly reversed MAP-induced hyperactivity at the doses of 0.01-0.1 mg/kg as shown in Figure 6A. In contrast, olanzapine

significantly decreased this hyperactivity in a dose-dependent manner as shown in Figure 6B ($P<0.01$; by Dunnett's multiple comparison test). We also evaluated the combined effects of ASP5736 and olanzapine on MAP-induced hyperactivity in mice. The inhibitory effect of olanzapine exhibited an approximate 3-fold increase in potency following the co-administration of ASP5736 (0.003 mg/kg, p.o.) (Figure 6C).

3.4.5. Effect of ASP5736 on catalepsy in mice

Existing antipsychotic drugs are known to induce catalepsy in rodents, with an index of extrapyramidal side effects (EPS). We therefore evaluated whether ASP5736 induces catalepsy in mice. ASP5736 did not induce catalepsy up to 3 mg/kg, p.o. (Figure 7A). In contrast, olanzapine dose-dependently increased the duration of catalepsy (Figure 7B). Further, the potency of olanzapine to induce catalepsy was not enhanced by 0.003 mg/kg (p.o.) of ASP5736, which had improved memory deficit in mice (Figure 7C).

3.4.6. Effect of ASP5736 on plasma prolactin level in mice

Existing antipsychotic drugs are known to increase the plasma prolactin levels in rodents, as well as in humans. We therefore evaluated if ASP5736 increases plasma prolactin levels in mice. ASP5736 did not influence plasma prolactin level at doses ranging from 0.001 to 1 mg/kg (p.o.) in mice as shown in Table 3. In contract, 0.1 mg/kg (p.o.) of risperidone, the drug which was evaluated as a positive control, significantly increased the plasma prolactin level (Table 3).

4. Discussion

Several other investigators (Grailhe et al., 2001, Francken et al., 1998, and Hurley et al., 1998) have confirmed that 5-HT inhibits adenylate cyclase activity and suppresses forskolin-induced cAMP accumulation by expressing the cloned 5-HT$_{5A}$ receptor in HEK-293 cells. And Noda et al. (2003) hypothesized that the activation of 5-HT$_{5A}$ receptor-induced IP$_3$ formation might in turn cause a transient increase in intracellular Ca^{2+} concentration. In support of this hypothesis, they reported a 5-HT-induced transient outward current in 5-HT$_{5A}$ receptor-expressing cells. Therefore, at first, we examined the in vitro profiles of our compound ASP5736. The compound antagonized the 5-CT-induced Ca^{2+} influx with an IC$_{50}$ value of 1.02 nM, being similar potency to its binding affinity to 5-HT$_{5A}$ receptor. Further, ASP5736 was shown to dose-dependently antagonize the 5-CT-induced decrease in cAMP in 5-HT$_{5A}$ receptor-expressing HEK293 cells. ASP5736 exhibits more than 200-fold selectivity for 5-HT$_{5A}$ receptor over other receptors, enzymes, channels, and other 5-HT subtypes, with the exception of human 5-HT$_{2c}$ receptor and 5-HT$_7$ receptor. From these results, we confirmed that ASP5736 is a novel, selective and potent 5-HT$_{5A}$ receptor antagonist.

Given the distribution of the 5-HT$_{5A}$ receptor mRNA (Plassat et al., 1992; Matthes et al., 1993; Erlander et al.,

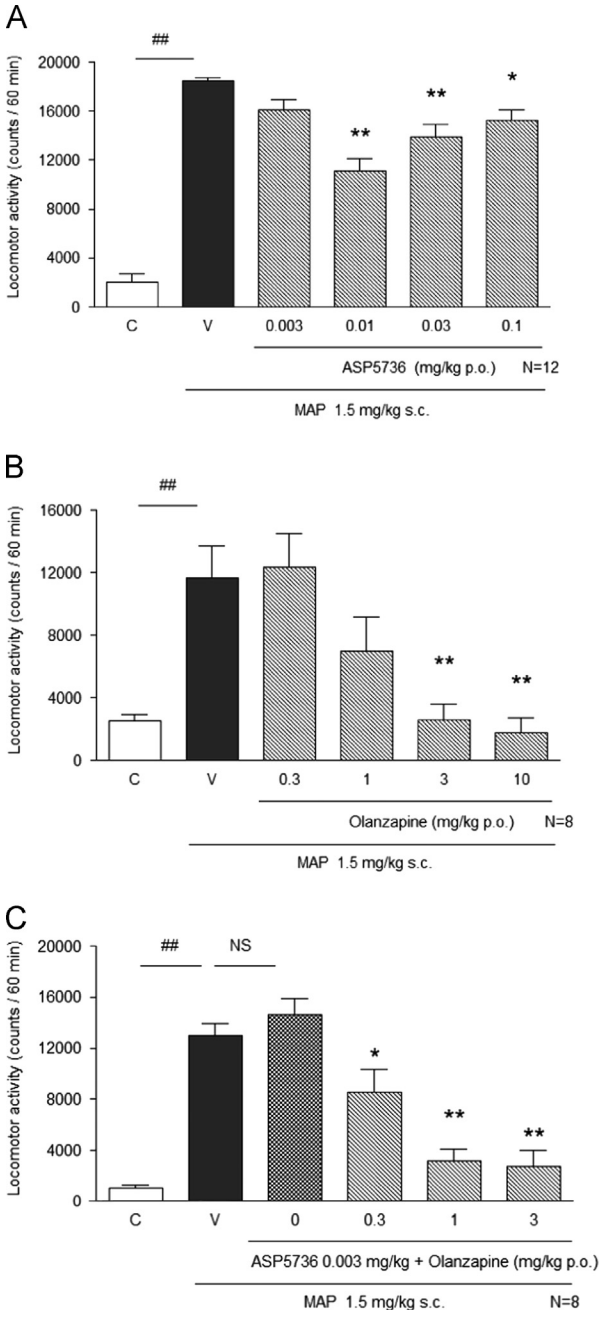

Figure 6 Effect of ASP5736 and olanzapine on MAP-induced hyperactivity in mice. (A) Effect of ASP5736 on MAP-induced hyperactivity. Each value indicates mean ± SEM ($n=12$ for each group). (B) Effect of olanzapine on MAP-induced hyperactivity. Each value indicates mean ± SEM ($n=8$ for each group). (A and B) $^{\#\#}P<0.01$, statistically significant compared with control (vehicle- and saline-treated) group (Student's t-test). $^{**}P<0.01$, statistically significant compared with vehicle-treated group (Dunnett's multiple comparison test). (C) Effect of olanzapine in combination with ASP5736 on MAP-induced hyperactivity. We determined the dose of ASP5736 from the effective dose of MK-801-Y-maze and nPCP-NORT study. control, saline- and vehicle-treated; MAP, MAP (1.5 mg/kg)- and vehicle-treated and ASP, MAP (1.5 mg/kg)- and ASP5736 (0.003 mg/kg)-treated. Each value represents mean ± SEM ($n=8$ for each group). $^{\#\#}P<0.01$, statistically significant compared with control (saline- and vehicle-treated) group (Student's t-test). $^{*}P<0.05$, $^{**}P<0.01$, statistically significant compared with MAP (1.5 mg/kg)- and ASP5736 (0.003 mg/kg)-treated group (Dunnett's multiple comparison test).

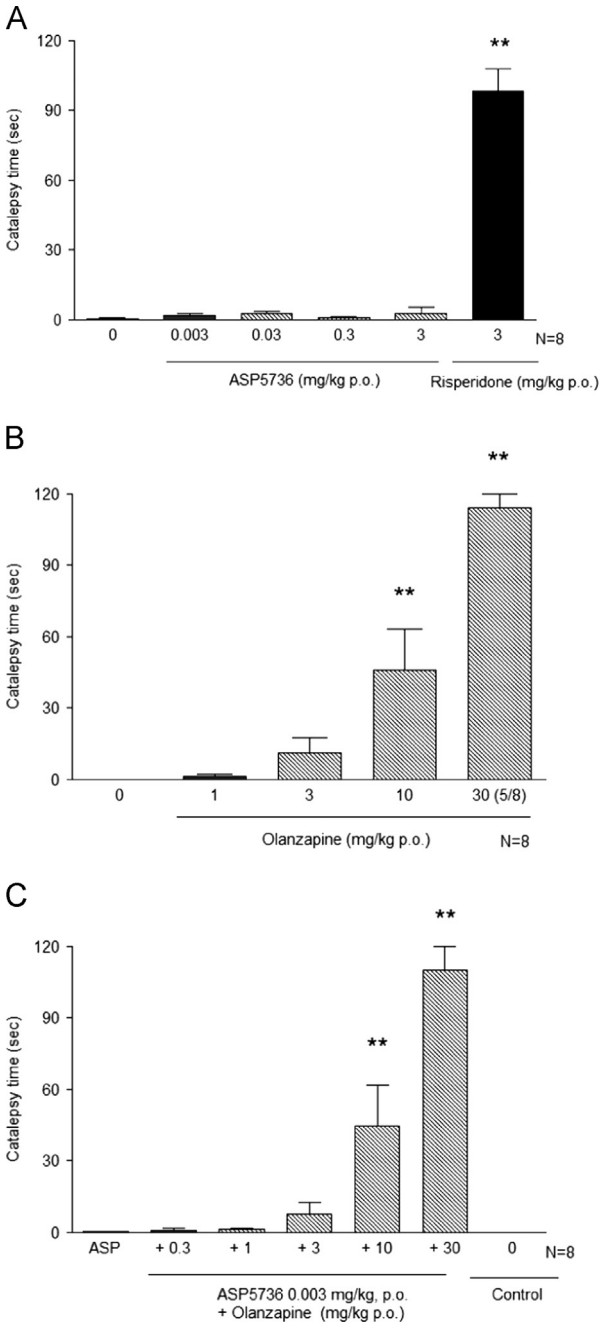

Figure 7 Effects of olanzapine and ASP5736 on catalepsy in mice Catalepsy time (s) (Max=120 s) of ASP5736, olanzapine and the effect of olanzapine with ASP5736 (0.003 mg/kg). (A) Effect of ASP5736 alone. ASP5736-induced catalepsy was not noted. (B) Effect of olanzapine alone. (C) Effect of olanzapine with ASP5736. Each value shows the mean±SEM. (n=8 for each group). **$P<0.01$, statistically significant compared with vehicle-treated group (Dunnett's multiple comparison test). For the combination of olanzapine and ASP5736, the dose of ASP5736 was determined from the effective dose of MK-801-Y-maze and nPCP-NORT study.

1993; Pasqualetti et al., 1998; Kinsey et al., 2001) and protein (Duncan et al., 2000; Oliver, Kinsey, 2000; Wang et al., 2000) and previous findings from analysis of 5-HT_{5A} receptor KO mice (Grailhe et al., 2001), we focused on

Table 3 Plasma prolactin level of ASP5736 in mice

	Dose (mg/kg, p.o.)	Plasama prolactin (ng/mL)
Vehicle	0	4.30±0.83
ASP5736	0.001	3.21±0.27
	0.01	5.18±1.29
	0.1	2.85±0.29
	1	5.26±2.06
Risperidone	0.1	33.23±3.71**

Plasma prolactin level of ASP5736 (positive control is risperidone 0.1 mg/kg at 0.5 h after oral administration) were measured at 1 h after oral administration of ASP5736 in mice. All data are expressed as mean±SEM (n=5 for each group).

**$P<0.01$, statistically significant compared with vehicle-treated group (by Dunnett's multiple comparison test).

schizophrenia as the therapeutic target of a selective 5-HT_{5A} receptor antagonist. One of the important findings of the present study was that ASP5736, but not olanzapine, ameliorated acute MK-801-induced working memory deficit in Y-maze (Marta et al. 2005; Olakunle et al. 2012) and neonatal-PCP-induced visual learning deficit in NORT, suggesting that ASP5736 could be beneficial for the cognitive deficits in putative animal models of cognitive deficits of schizophrenia.

The discovery that noncompetitive NMDA receptor antagonists PCP, MK-801 and ketamine induce psychotic reactions in adult human subjects that resemble schizophrenia symptoms led to the theory that glutamatergic neuronal dysfunction is involved in the etiology and pathophysiology of schizophrenia (Javitt and Zukin 1991). A single treatment of MK-801 or chronic treatment with PCP in adult rodents induced cognitive impairment in NORT (Kunitachi et al. 2009). Neonatal treatment with PCP induces an increase in spontaneous motor activity, impairment of prepulse inhibition, and behavioral changes with regard to learning and cognition (Andersen and Pouzet 2004; Depoortere et al. 2005) as well as apoptotic neurodegeneration in adult rats, symptoms associated with the pathology of schizophrenia (Wang et al. 2001). The current study confirms that neonatal treatment with PCP does indeed induce cognitive impairment in NORT in mice on maturation into adults. Furthermore, neonatally PCP-treated mice, which had been treated with PCP on postnatal days 7, 9 and 11, were used in the NORT at 8-9 weeks old. As PCP does not remain in the body at the time of NORT, we don't think that the effects observed with ASP5736 reflected mere pharmacological reversal, at least in this model.

At present, we are examining the mechanism by which ASP5736 exerts efficacy in various animal models of cognitive impairment using microdialysis, immunohistochemistry, and microarray studies. We recently discovered (Yamamoto et. al, unpublished observation) by immunostaining that 5-HT_{5A} receptors are co-expressed on DA neurons (TH-positive) in VTA (ventral tegmental area). We therefore hypothesize that ASP5736 in VTA might block the inhibitory 5-HT_{5A} receptor on

DA neurons projecting to the mPFC (medial prefrontal cortex) and thereby improve cognitive impairment by activating DA neurons in the mPFC. The results will be published elsewhere after completing studies.

Add-on studies with olanzapine in our present study have revealed a number of findings. Although olanzapine failed to improve MK-801-induced spontaneous alternation behavior deficit, ASP5736 with olanzapine significantly attenuated such behavior to the same degree as ASP5736 administered alone. Further, the sedative effect of olanzapine was not enhanced by ASP5736 at 0.003 mg/kg, which is an effective dose against MK-801-induced spontaneous alternation deficit. MAP-induced hyperactivity (positive-like symptom model) was also found to be significantly ameliorated by ASP5736 alone, and the inhibitory effect on MAP-induced hyperactivity of olanzapine increased potency by approximately three-fold following administration of ASP5736. The catalepsy liability of olanzapine was also not affected by 0.003 mg/kg of ASP5736, a dosage which improved memory deficit in mice. Taken together, these findings suggest that ASP5736 alone significantly ameliorated MAP-induced hyperactivity at the same dosage as in combination with olanzapine, it is possible that it works via a distinct mechanism from olanzapine. Of note, the adverse effects (sedation and catalepsy) of olanzapine were not worsened by the co-administration of ASP5736.

Here, we clearly indicate for the first time that ASP5736 is a novel and potent 5-HT$_{5A}$ receptor antagonist that can improve positive-like symptoms and cognitive impairment in animal models of schizophrenia without adverse effects. Of further note, these effects are not reduced on co-administration with the existing antipsychotic olanzapine, suggesting that ASP5736 may satisfy current unmet medical needs for the treatment of schizophrenia and also that the compound can be treated in combination with existing antipsychotics like olanzapine.

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Contributors

Management of research design: Yamazaki, Harada, Ni and Matsuoka. Conducted experiments: Yamazaki, Okabe, Yarimizu, Yamamoto, Shimada. Wrote the first draft of the manuscript: Yamazaki. Contributed to the writing of the manuscript: Yamazaki, Okabe, Yarimizu, Yamamoto, Shimada, Harada, Ni and Matsuoka. All authors contributed to the present studies and approved the final manuscript.

Conflict of interest

We do not have any conflicts of interest in connection with this manuscript.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.euroneuro.2014.07.009.

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